Cell-specific measurement of cytosolic glutathione in poplar leaves*

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ABSTRACT

The level of glutathione (GSH) in plants is important in defence reactions against biotic and abiotic stresses and can place considerable demand of the sulphur assimilation pathway. Enzymes involved in sulphur assimilation and GSH metabolism are not evenly distributed between different subcellular compartments or between different cell types in leaves or roots; however, there is little information on the effect that such asymmetries have on the actual GSH concentration in each compartment or cell type. In the present study in situ labelling with monochlorobimane (MCB) in combination with confocal laser scanning microscopy was used to quantify GSH in each of the main cell types of poplar leaves from fluorescence of the GSB conjugate formed. Comparison of results from the in situ approach with total GSH levels measured in vitro by high-performance liquid chromatography suggested that only the cytosolic GSH pool was labelled using this approach. This suggests that an appropriate GST was not present within the chloroplasts to catalyse the conjugation reaction and that chloroplastic GSH does not rapidly exchange with the cytoplasmic pool under the conditions of the assay. Cytosolic GSH levels were between 0.2 and 0.3 mM for both photosynthetic and non-photosynthetic (epidermal) cell types in wild-type poplar leaves. Cytosolic levels increased by around two-fold in transgenic poplars over-expressing activities of GSH biosynthesis including the detoxification of active oxygen species (AOS), xenobiotics and heavy metals (Noctor & Foyer 1998; May et al. 1998, Noctor et al. 1998a; Cobbett 2000). GSH is synthesized in two steps from its constituent amino acids cysteine, glutamate and glycine. First, the γ-glutamyl bond is formed between glutamate and cysteine in a reaction catalysed by γ-glutamylcysteine synthetase (γ-GCS). In the second reaction glycine is added to the C-terminal end of γ-glutamylcysteine (γ-EC) by glutathione synthetase (GSHS) to form GSH. Unlike enzymes involved in the reduction of sulphate, enzymes for the incorporation of sulphide into cysteine and the two-step pathway for GSH biosynthesis are present in both cytosol and the stroma of the plastids (Saito 2000; Noctor et al. 2002). As sulphate reduction takes place exclusively in the plastids, there must be a substantial net export of reduced sulphur from the chloroplast to cover the demand for reduced sulphur in the cytosol, particularly under conditions favouring further export of GSH to other tissues (Herschbach et al. 2000) or in response to depletion of GSH under stress conditions. At present it is not known to what extent that HS−, γ-EC or GSH contribute to this

INTRODUCTION

Compartmentalization of metabolism between different organs, different cell types within one tissue and between different subcellular compartments is a general feature of plant physiology and an important component of metabolic regulation (Bowsher & Tobin 2001). A well-understood pathway with distinct subcellular compartmentalization is the assimilation of sulphate. In this pathway the reduction of adenosine S′-phosphosulphate (APS) by APS reductase and the subsequent reduction of sulphite to sulphide by sulphite reductase (SiR) take place exclusively in the plastids (Leustek et al. 2000). In photosynthetic cells, the light reactions provide ample reducing equivalents, in non-photosynthetic tissue or in darkness, the NADPH is produced from the oxidative pentose phosphate pathway (OPPP). Following reduction, sulphide immediately gets incorporated into cysteine, which is a precursor for the biosynthesis of methionine, proteins and the tripeptide glutathione. Reduced glutathione (GSH) is the most abundant low molecular weight thiol and GSH serves as a mobile pool for long-distance transport of reduced sulphur between different organs and as a reservoir of reduced sulphur that can be re-mobilized under increased demand (Rennenberg 2001). Due to its nucleophilic activity GSH also plays an essential role in various stress–response pathways including the detoxification of active oxygen species (AOS), xenobiotics and heavy metals (Noctor & Foyer 1998; May et al. 1998, Noctor et al. 1998a; Cobbett 2000).

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* Dedicated to Prof. Dr Ludwig Bergmann on the occasion of his 75th birthday.

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export, nor has the identity of the transporter(s) been resolved. More recently, it has been shown that isolated chloroplasts are capable of active uptake of GSH (and possibly the oxidized form, GSSG) through two putative transport systems with differing affinities (Noctor, Veljovic-Jovanovic & Foyer 2000, Noctor et al. 2002), and it has been suggested that these may operate to equilibrate GSH concentration between chloroplasts and cytosol or assist in maintaining a highly reduced GSH pool through rapid recycling of oxidized GSSG back to the chloroplast (Noctor et al. 2002). In addition to intracellular co-operation between different compartments, exchange between different cell types may also contribute to the maintenance of resting GSH levels. Thus, certain cell types, such as bundle-sheath cells in C₄ plants, which have limited photosystem II activity, do not have sufficient NADPH to sustain high rates of GSSG reduction, and are dependent on transport from the mesophyll cells to establish and maintain a reduced GSH pool (Douls et al. 1997; Noctor et al. 2002). At this stage it is not clear whether similar co-operation occurs with the non-photosynthetic cells in the leaf, such as epidermal cells, that would have to rely primarily on OPPP for NADPH production for S-assimilation, GSH biosynthesis and GSSG reduction. Differential levels of GSH are maintained in certain cell types, such as trichomes (Gutiérrez-Alcalá et al. 2000), and it is likely that levels in stomatal guard cells are independently regulated as they are sympatrically isolated from the rest of the leaf.

The factors that regulate the cytosolic and chloroplastic concentration of GSH are beginning to be dissected using transgenic plants over-expressing key enzymes in the biosynthetic pathway. For example, cytosolic over-expression of bacterial γ-ECS in poplars, mustard or tobacco led to significantly increased overall GSH levels (Noctor et al. 1996; Zhu et al. 1999; Pilon-Smits et al. 1999; Creissen et al. 1999). In poplars over-expression of bacterial γ-ECS enhanced GSH levels about two- to four-fold and γ-ECS levels up to 15-fold without depletion of cytosolic pools (Noctor et al. 1996; Arisi et al. 1997). Over-expression of bacterial γ-ECS in the plastids has also been reported for tobacco (Creissen et al. 1999), Brassica juncea (Pilon-Smits et al. 1999; Zhu et al. 1999) and poplars (Noctor et al. 1998b) with concomitant increases in total extractable GSH. Interestingly, poplars over-expressing γ-ECS in the cytosol are more tolerant towards chloracetalddehyde herbicides than poplars over-expressing γ-ECS in the plastids (Gullner, Kümves & Rennenberg 2001), suggesting that biosynthesis of GSH in the plastids cannot sufficiently supply the cytosol under conditions of high demand.

In this paper we set out to determine first, whether GSH is evenly distributed throughout each cell type in mature poplar leaves using in situ labelling technique and confocal laser scanning microscopy (CLSM) (Meyer, May & Fricker 2001), second, whether this technique can deliver information about the subcellular distribution of GSH, and third, whether over-expressing γ-ECS in the cytosol drives differential changes in GSH concentration in different cell types or between the cytosol and chloroplasts.

**MATERIALS AND METHODS**

**Plant material**

Experiments were performed with 7–9 week old wild-type (WT) and transgenic poplar trees (Populus tremula × P. alba, INRA Clone 717 1B4; Leple et al. 1992; Arisi et al. 1997). From a collection of different transgenic lines over-expressing γ-ECS from Escherichia coli in the cytosol line ggs28 was used, because this line shows strong expression of γ-ECS and contains very high quantities of GSH (Noctor et al. 1997). Plants were micropropagated as sterile cultures. After root formation the plants were transferred to a soil mixture and grown in a greenhouse under a 16 h light : 8 h dark regime. The soil mixture consisted of 1 part sand, particle size 0.06–0.2 mm, 1 part sterilized commercial soil and 2 parts Perlite (Agripierl; Perlite-Dämmstoff-GmbH, Dortmund, Germany). The trees were supplied every 2 weeks with 200 mL commercial fertilizer (Hakaphos blau, COMPO GmbH, Münster, Germany; 3 g L⁻¹, 15% N, 10% P₂O₅, 15% K₂O, 2% MgO, 0.01% B, 0.02% Cu, 0.05% Fe, 0.05% Mn, 0.001% Mo and 0.015% Zn).

For labelling of isolated intact plastids fresh spinach (Spinacia oleracea L) plants were obtained from the local market.

**Fluorescent dyes and labelling of leaf pieces**

In situ labelling of GSH was carried out with MCB as fluorescent marker. Stock solutions of 100 mM MCB (Molecular Probes, Eugene, OR, USA) were prepared in dimethyl sulphoxide (DMSO) and stored at −20 °C. Aliquots were thawed immediately prior to use and diluted in deionized water to a final concentration of 100 µM. Propidium iodide (PI) intercalates cell wall polymers and nucleic acids, which causes a strong increase in fluorescence. However, because PI (Molecular Probes) is membrane impermeable it is excluded from intact cells and can be used as an indicator for cell viability to distinguish viable and dead cells. PI was prepared and added to the dye solution at a final concentration of 50 µM. Sodium azide (Sigma-Aldrich, Taufkirchen, Germany) was freshly prepared and added to the dye solution at a final concentration of 5 mM to deplete ATP levels and thereby inhibit vacuolar sequestration of glutathione S-bimane conjugate (GSB).

Epidermal cells and spongy mesophyll cells were imaged from the upper or the lower leaf side, respectively. GSH in palisade mesophyll cells was measured from free-hand cross-sections of the leaves. Imaging from the lower side required removal of trichomes by cutting them off with a razor blade to get good access to the leaf surface. Small leaf pieces of about 3 mm × 3 mm width and cross-sections were cut out of the third intercostal area of the 10th leaf counted from the apex. Leaf pieces and cross-sections were both infiltrated with the appropriate dye solution by applying vacuum for 10 min and incubated for 60 min at room temperature.

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Measurement of GSB levels with confocal laser scanning microscopy

After a total incubation time of 60 min at room temperature leaf pieces and cross sections were transferred to a drop of deionized water on a microscope slide and covered with a cover-slip using adhesive tape about 150 μm thick as spacer between slide and cover-slip to avoid squashing of the leaf pieces. Intact cells were imaged using either a MRC-1024 (Bio-Rad Microscience Ltd, Hemel Hempstead, UK) or a LSM510 system (Zeiss, Jena, Germany). The MRC-1024 was attached to a Nikon Diaphot (Nikon UK Ltd, Kingston-upon-Thames, UK) inverted microscope equipped with a Nikon 60 x 1.4 NA Plan-Apochromat oil-immersion lens. Excitation was achieved with a 442 nm HeCd laser (Liconix, Santa Clara, CA, USA) coupled into the scan head by a fibre-optic. Fluorescence of GSB was recorded at \( \lambda_{em} = 522 \pm 35 \text{ nm} \) and PI fluorescence at \( \lambda_{em} > 585 \text{ nm} \) with a long pass filter. The LSM510 was mounted on an inverted microscope (Axiovert 100 M; Zeiss, Jena, Germany) and a C-Apochromat 40 x 1.2 NA water immersion lens (Zeiss) was used for imaging. In this case fluorescence was excited at 364 nm (Enterprise UV Laser; Coherent, Dieburg, Germany). Emission of GSB fluorescence was recorded at \( \lambda_{em} = 505–530 \text{ nm} \) and emission of PI fluorescence was collected at \( \lambda_{em} > 560 \text{ nm} \) with a long-pass filter.

To determine the average cytosolic GSH level the fluorescence intensities were routinely measured in the nuclei of the different cell types after conjugation of GSH to MCB. Fluorescence was then converted to average cellular GSB levels using the \textit{in vitro} GSB calibration (see below). In those cells with larger continuous volumes of cytoplasm measurements of fluorescence were also conducted in these areas. However, no difference in fluorescence intensity was found between cytosol and nuclei. For images taken through the epidermal cell layers the fluorescence intensity for each cell was corrected for attenuation using empirically determined depth-dependence correction factors.

To follow the labelling kinetics over time, slides with leaf pieces were placed on the microscope stage immediately after vacuum infiltration and optical \((x,y)\) sections of epidermal cells were collected with a sampling rate of 5 min over 50–110 min. The mages were Kalman averaged over four frames.

Calibration of GSB fluorescence

A 10 mM stock solution of GSB was made from 10 mM MCB and 100 mM GSH in the presence of glutathione S-transferase (Rabbit Liver GST, G 8216; Sigma-Aldrich). Three or four different concentrations of GSB ranging from 0.1 to 1 mM were routinely used for calibration. Fluorescence of GSB in intact cells was calibrated against GSB standard imaged using identical instrument settings.

Depth-dependent attenuation correction

For attenuation correction of the loss of fluorescence signal with increasing depth into the tissue, leaf pieces were infiltrated with a solution containing 100 μM MCB, 50 μM PI, 5 mM azide and 1 mM GSB. After incubation serial optical sections were collected with excitation at 442 nm and a z-step motor increment of 2 μm. Average GSB intensities were measured in intercellular spaces in 2 μm steps from the upper and the lower surface of the leaf to a depth of about 40 μm into the tissue. The average loss of signal intensity was plotted against the depth into the tissue to determine the attenuation of the signal for the upper and the lower side of the leaf, respectively. Additionally, intracellular fluorescence was compared to the fluorescence from the 1 mM GSB solution infiltrated into the intercellular spaces as an internal standard, which could be measured at the same depth as the nuclei in the investigated cells.

Auto-fluorescence and GSH-depletion controls

For determination of auto-fluorescence leaf pieces were vacuum infiltrated with 50 μM PI and 5 mM sodium azide and incubated for at least 1 h. Images were taken as described above. 1-Chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich) was used to compete out the labelling of GSH with MCB. In this case leaf pieces were infiltrated with a solution containing 10 mM CDNB, 5 mM azide, 50 μM PI and 100 mM MCB and incubated for at least 1 h. Afterwards samples were used for imaging or high-performance liquid chromatography (HPLC) analysis, respectively. CDNB was stored as a 1 M stock solution in DMSO.

HPLC analysis of GSH

For the analysis of bimane-labelled low molecular weight thiols, leaf pieces were vacuum infiltrated with 100 μM MCB and 5 mM sodium azide for 10 min and incubated at room temperature. At different time points after infiltration leaf pieces were removed from the dye solution and ground on ice with a pestle and mortar in 1 mL 200 mM methansulphonic acid (MSA, Sigma-Aldrich). The extract was centrifuged for 10 min at 4 °C at 12000 × g and an aliquot of the supernatant was subjected to HPLC analysis as described previously (Schupp & Rennenberg 1992). To compare the \textit{in situ} labelling method with previously published data, low molecular weight thiols were additionally analysed in leaf extracts using monobromobimane (MBB; Thiolyte®MB; Calbiochem, Bad Soden, Germany) as described before (Herschbach et al. 2000). In a further approach leaf pieces were first infiltrated with MCB in the presence of azide as described above. After 2 h incubation leaf pieces were taken out of the dye solution, washed and ground in 1 mL 0.1 N HCl. Samples were centrifuged at 4 °C and 12000 × g and an aliquot of the supernatant was taken for additional \textit{in vitro} analysis of thiols that had not been labelled \textit{in situ}. This post-extraction labelling was done with MBB as described above.

Chloroplast isolation and thiol labelling

About 20 g of finely chopped leaf tissue was homogenized in 200 mL of semi-frozen isolation medium (0.33 M sorbitol,
10 mM Na₄(PO₄)₂-HCl, pH 6.5, 5 mM MgCl₂, 2 mM ascorbate). The homogenate was filtered through four layers of Miracloth (Calbiochem). The unfractionated filtrate was centrifuged 2 min at 4 °C and 4330 × g and the pellet was re-suspended in re-suspension medium (0.33 M sorbitol, 50 mM Hepes-KOH, pH 7.6, 1 mM MgCl₂, 1 mM EDTA). The suspension was loaded on top of 10 mL of re-suspension medium containing 50% (v/v) Percoll. Chloroplasts were pelleted by centrifugation at 3000 × g and 4 °C for 5 min and re-suspended in re-suspension medium. Fifty microlitres of the chloroplast suspension was labelled with 100 µM MCB for 30 min, ground in 1 mL 200 mM MSA and analysed by HPLC as described above. For determination of total low molecular weight thiols 50 µL chloroplasts were extracted in 1 mL 0.1 N HCl, centrifuged at 12000 × g at 4 °C for 10 min and an aliquot of the supernatant was subjected to thiol labelling with MBB and HPLC analysis as described above. Soluble protein in chloroplast suspensions was measured according to Bradford (1976). Chlorophyll content was determined photometrically (Lichtenthaler & Wellburn 1983).

Software

Image processing and analysis was performed with the software packages Scion Image® (Scion Corp., Frederick, MD, USA), Confocal Assistant® (T.C. Brelje, University of Minnesota, USA), Zeiss LSM Image Examiner (Version 2.30.011; Zeiss) and Photoshop® (Adobe Systems Inc., San Jose, CA, USA). Graphical analysis was performed with Excel® (Microsoft Corp., Seattle, WA, USA). Statistical analysis was performed using t-test and the one-factorial analysis with SPSS for Windows (Release 9.0, SPSS Inc., Chicago, IL, USA).

RESULTS

GSH is specifically labelled by MCB in all cell types of mature poplar leaves

Labelling pieces of mature poplar leaves with MCB resulted in strong blue-green fluorescence in the cytoplasm following conjugation to GSH to form fluorescent glutathione S-bimane (GSB) in every cell type examined (Fig. 1a. d. f & h). In most experiments, the GSB formed was restricted to the cytosol and the nuclei by inclusion of sodium azide to deplete ATP levels and block ATP-dependent vacuolar sequestration of GSB. In the absence of azide, in most cells GSB was rapidly transferred to the vacuole, completely clearing the cytoplasm (compare Fig. 1g & h). Only some cells close to the cut surfaces showed inhibition of vacuolar sequestration although labelling in the cytoplasm appeared to be similar to cells more distant from the cuts (not shown).

Virtually all the cells remained intact during the infiltration and labelling procedure as judged by the exclusion of the membrane-impermeant mortal dye propidium iodide (PI). Damaged cells were clearly identified by strong red fluorescence of the nucleus and were excluded from further analysis. Conveniently PI also labelled the cell walls in intact cells, thus providing an outline of the cells. The additional red spots in the cytoplasm of mesophyll cells and guard cells were due to auto-fluorescence from chlorophyll.

In the absence of MCB, levels of cytoplasmic auto-fluorescence were negligible with excitation at 442 nm (e.g. Fig. 1b & i). When fluorescence was excited with UV light at 364 nm some mesophyll cells adjacent to the upper and lower epidermal cell layer showed varying levels of blue-green auto-fluorescence in the vacuoles. MCB in the presence of sodium azide still gave strong cytoplasmic labelling in these cells (Fig. 1d & e) and the spatial resolution of the system was sufficient to separate the signal from the nucleus from the vacuolar auto-fluorescence.

To test whether labelling in situ was limited to GSH, CDNB, a well-known model substrate for GSTs that gets rapidly conjugated to GSH, was included in the assay to deplete GSH levels. At 10 mM, CDNB abolished all MCB-dependent fluorescence (Fig. 1c). The absence of fluorescent signal would be consistent with specific labelling of GSH and not with other low molecular weight thiols or proteins.

To further test that the labelling was specific for GSH, leaf pieces incubated with 100 µM MCB in the presence of 5 mM sodium azide were extracted and the extracts subjected to HPLC analysis of the fluorescently labelled low molecular weight thiols. This analysis unambiguously showed the high specificity of the labelling for GSH (Fig. 2a). In the absence of azide, when vacuolar transport was allowed to take place, additional minor peaks for cysteine, cysteinylglycine and γEC were found (data not shown). After simultaneous incubation of leaf pieces with 100 µM MCB and 100-fold excess of CDNB only a small amount of GSH remained available for conjugation to MCB (Fig. 2a & b).

Although the data presented above indicate that the in situ labelling approach was specific to GSH, it is only possible to infer the resting levels of GSH by this approach if all the accessible GSH is conjugated (Meyer et al. 2001). Typically in situ labelling follows a progress curve for conjugation of MCB to GSH by GST(s). To identify the time point at which the accessible GSH pool was completely labelled, the progress curve for labelling was followed both by time-lapse CLSM and also by HPLC after extraction of labelled tissue at different times after the start of the labelling. The fluorescence intensity rapidly increased in all cells of the leaf. For example, Fig. 3a shows the increase of MCB-dependent fluorescence in epidermal cells at different time points. The increase of fluorescence intensity in the cytosol followed a saturation curve and reached a plateau after about 60 min (Fig. 3b). The plateau value reached after 60 min was similar for all cells, independent of their distance from the wound surface, although the rate of labelling appeared to be slightly reduced in cells close to the wound. Time courses for the increase of the GSB peak in HPLC profiles gave similar results, with the amount of labelled GSH extracted increasing until about 60 min after start of
Figure 1. In situ localization of glutathione in different cell types of live poplar leaves. Small pieces from poplar leaves were vacuum infiltrated with dye solution and images were taken by confocal laser scanning microscopy after an incubation period of 1 h. (a) Cells of the upper epidermis labelled with 50 μM propidium iodide (PI) and 100 μM monochlorobimane (MCB) in the presence of 5 mM sodium azide to deplete ATP levels and thereby inhibit vacuolar sequestration of GSB. λex = 442 nm. (b) Cells of the upper epidermis labelled with 50 μM PI showing the cell walls and confirming the absence of green autofluorescence in the cytoplasm. Absence of nuclear labelling with PI confirmed integrity of the plasma membrane. λex = 442 nm. (c) Cells of the upper epidermis labelled with 50 μM PI and 100 μM MCB in the presence of 5 mM sodium azide and 10 mM 1-chloro-2,4-dinitrobenzene. λex = 442 nm. (d) Cross-section of a leaf, labelled with 50 μM PI and 100 μM MCB in the presence of 5 mM sodium azide. λex = 364 nm. (e) Cross-section of a leaf, stained with 50 μM PI in the presence of 5 mM sodium azide. λex = 364 nm. (f) Guard cells on the lower epidermis, stained with 50 μM PI and 100 μM MCB in the presence 5 mM sodium azide. λex = 442 nm. (g) Spongy mesophyll cells, labelled with 50 μM PI and 100 μM MCB. λex = 442 nm. (h) Spongy mesophyll cells, labelled with 50 μM PI and 100 μM MCB in the presence of 5 mM sodium azide. λex = 442 nm. (i) Spongy mesophyll cells, labelled with 50 μM PI. λex = 442 nm.
labelling and then remaining stable between 60 and 120 min (Fig. 3b).

**MCB labels only cytosolic GSH in poplar leaves**

To investigate whether MCB labelled the entire cellular GSH pool or only a fraction of this pool, the in situ-labelled tissue was extracted after fluorescence reached the plateau. This extract was then subjected to a conventional thiol labelling protocol including reduction of disulphides with dithiothreitol (DTT) and labelling of thiols with MBB. In this way it was expected to label any remaining GSH that was not labelled with MCB in situ. In WT poplars 98.8 ± 33.0 nmol GSH g⁻¹ fresh weight (FW) were labelled in situ (Fig. 4). This equates to 50 ± 17% of the total GSH pool measured after subsequent in vitro labelling of remaining GSH in the leaf extracts. The accessible GSH pool labelled in the transgenic poplar leaves was roughly two-fold higher than in the WT (Fig. 4). Subsequent in vitro labelling with MBB resulted in a further increase in GSH labelling, but the absolute difference between the amount of GSH labelled in situ and the total amount of GSH was comparable with the difference found in WT leaves. The
imaging experiments clearly showed that the cytosolic pool was labelled, thus, the simplest explanation of this data is that a substantial second pool of GSH was present, most probably in the plastids, but was not accessible to \textit{in situ} labelling by low (100 \textmu M) concentrations of MCB. Furthermore, the size of this pool was not affected by cytosolic over-expression of \textit{g}-ECS.

The hypothesis that MCB did not label the chloroplastic GSH pool was further corroborated by the observation that chloroplasts were negatively stained after \textit{in situ} labelling with 100 \textmu M MCB in the presence of azide. For example, despite the strong signal from the cytosol and nucleus in guard cells, completely clear regions remain that correspond to the position of the chloroplasts (Fig. 5a & b). The lack of labelling is most likely due to the absence of an appropriate GST in the chloroplasts rather than lack of penetration of the highly membrane-permeant MCB dye.

To test this hypothesis we tried to isolate intact chloroplasts from poplar leaves and then compare labelling with monobromobimane (MBB), which is more reactive than MCB and does not require a GST for conjugation. Unfortunately, however, attempts to isolate sufficient intact chloroplasts from poplars were not successful, so we studied GSH labelling in isolated chloroplasts from spinach. Whereas the addition of 100 \textmu M MCB to a chloroplast suspension with >80\% intact chloroplasts resulted only in minor labelling, the addition of the far more reactive MBB resulted in enhanced labelling. In this case the amount of labelled GSH was similar to the amount of GSH labelled after osmotic rupture of the chloroplast envelope (Fig. 5c).

Taken together, all these results are consistent with labelling of only the cytosolic GSH pool during the \textit{in situ} assay with 100 \textmu M MCB, rather than labelling total cellular GSH.

### Figure 4.

Comparison of the amount of glutathione labelled \textit{in situ} with total glutathione. Leaf pieces of wild-type (WT) and transgenic poplars over-expressing \textit{g}-glutamylcysteine synthetase in the cytosol (line \textit{ggs}28) were infiltrated with 100 \textmu M monochlorobimane and 5 \textmu M sodium azide. Labelled thiols were extracted after 120 min incubation and directly analysed by HPLC (i.e. \textit{in situ} labelled glutathione; filled bars) or extracted thiols were additionally reduced with DTT, derivatized with monobromobimane and then analysed by HPLC (i.e. total glutathione; open bars). Bars represent mean ±SD of at least three independent experiments.

### Cytosolic GSH levels are uniform in all cell types for mature poplar leaves and respond similarly to over-expression of \textit{g}-ECS in the cytosol

Having ascertained that the \textit{in situ} assay was specific for cytosolic GSH levels, we set out to determine using CLSM whether there was variation in GSH concentration between different cell types within the leaf. When focusing deeper into the tissue, the amount of fluorescence decreased due to progressive blurring of excitation and/or emission light and consequent rejection of the out-of-focus light at the confocal pinhole. Thus, quantitative measurements of the GSB fluorescence required compensation for depth-dependent attenuation of fluorescence. To obtain an estimate of the

### Figure 5.

Monochlorobimane (MCB) does not label chloroplastic glutathione. (a, b) Direct visualization of subcellular distribution of glutathione S-bimane (GSB) in guard cells after infiltration of leaf pieces with 100 \textmu M MCB, 50 \textmu M propidium iodide (PI) and 5 \textmu M sodium azide. Green MCB-dependent fluorescence \((\lambda_{em} = 505–535 \text{ nm})\) is only visible in the cytosol, excluding the chloroplasts (arrows) (a). The image collected on the second detector \((\lambda_{em} > 585 \text{ nm})\) shows PI-dependent fluorescence exclusively located in cell walls and red autofluorescent spots in the cytosol clearly identifying the negatively contrasted compartments in the GSB image as chloroplasts (b). (c) Labelling of glutathione in isolated chloroplasts from spinach with MCB or monobromobimane (MBB), respectively. Intact chloroplasts were incubated with 100 \textmu M MCB or MBB for 60 min and labelled thiols subsequently analysed by HPLC. For comparison isolated chloroplasts were broken by osmotic shock and extracted. The low molecular weight thiols were reduced with DTT and subsequently derivatized with MBB. The diagram shows mean values ±SD of two to five independent experiments. Different letters indicate significant differences between treatments \((P \leq 0.05)\).
degree of attenuation, leaf pieces were infiltrated with a solution containing 1 mM GSB in addition to MCB, PI and sodium azide. Because GSB cannot enter intact cells and therefore remains in the intercellular spaces after infiltration, the signal intensity could be measured in the apoplast from horizontal \((x, y)\) sections collected at different depth into the tissue. Average fluorescence intensities were normalized to the top of the sample, plotted against the depth of the tissue and then fitted with a square function (Fig. 6). For both upper and lower side of the leaf the measured fluorescence intensity decreased with increasing depth. However, whereas for the epidermal cell layer on both sides of the leaf the attenuation of the fluorescence was similar, the decrease in fluorescence intensity in the spongy mesophyll was less than in the palisade parenchyma. For this reason separate attenuation corrections were applied to images collected from either the upper or the lower side of the leaf, respectively. The inverse of the attenuation values were applied as correction factors to the original images collected at the same depth.

Infiltration with 1 mM GSB revealed that cross-sections showed no significant loss of signal between the surface of the cut and the first intact cell layer, which was used for the quantitative measurements of \([GSH]_{\text{cyt}}\) in mesophyll cells.

Quantitative analysis of GSB fluorescence in spongy mesophyll cells showed that fluorescence intensities measured from images collected through the epidermal cell layer and corrected for attenuation were similar to values obtained from cross-sections without further correction (e.g. line ggs28: corrected 0.64 ± 0.36 mM, directly measured 0.69 ± 0.19 mM).

To investigate the distribution of GSH within the leaf, GSH levels were measured in different cell types across the leaf following depth-dependent correction of signal attenuation and calibration to give average cytosolic GSB concentrations, which in turn was taken as a measure of \([GSH]_{\text{cyt}}\). The average \([GSH]_{\text{cyt}}\) was about 0.2-0.3 mM in all cell types investigated in the WT poplar leaves with no significant differences between the different cell types (Fig. 7). The average \([GSH]_{\text{cyt}}\) in different cell types of the transgenic line was about 0.6 mM (Fig. 7) and was consistently two- to three-fold higher than in the equivalent cell types in WT leaves. This compares well with the difference in total GSH content of transgenic leaves, which was 2.5-fold higher than in WT leaves (1051 ± 306 nmol g\(^{-1}\) FW compared with 415 ± 89 nmol g\(^{-1}\) FW). Similar to the WT leaves, no significant differences in \([GSH]_{\text{cyt}}\) across the different cell types were found, however, there was considerable variability in the overall increase in \([GSH]_{\text{cyt}}\) between different trees that led to larger error bars on these measurements.

**DISCUSSION**

Here we show that the fluorescent *in situ* labelling of GSH with MCB combined with CLSM can be used to specifically measure GSH in different cell types of poplar leaves and that in poplar the *in situ* labelling reports cytosolic GSH.
rather than total GSH. The in situ assay used requires due attention to the kinetics and specificity of MCB labelling in situ (Fricker & Meyer 2001) and pragmatic correction for the optical aberrations inherent in imaging living tissues (White et al. 1996; Fricker et al. 2000). Inclusion of sodium azide to deplete ATP levels served to block both de novo biosynthesis of GSH that might occur in response to depletion by conjugation to MCB (e.g. Meyer & Fricker 2002) and ATP-dependent vacuolar sequestration of GSB via a GS-X conjugate pump (Coleman, Randall & Blake-Kalff 1997; Rea et al. 1998; Fricker & Meyer 2001) that might lead to further metabolism of the conjugate (e.g. Lamoureux & Rusness 1993; Wolf, Dietz & Schroeder 1996).

Time course measurements confirmed that the progress curve for the in situ labelling in the presence of azide followed a saturation kinetic and reached a plateau after about 60 min. Although the leaf tissues had to be cut to get good access to the different mesophyll cells this approach did not affect the plateau values reached after 60 min of incubation with MCB. Furthermore comparison of data obtained from measurement of mesophyll cells in cross-sections and hence cells close to the wound surface with GSH values for the same cell types measured by imaging through the epidermal cell layer of larger leaf pieces, suggested that possible wound effects did not significantly affect the plateau value of labelling used for quantification of cytosolic GSH. Extraction and subsequent HPLC analysis of low molecular weight thiols confirmed specificity of the labelling for GSH. The retention of GSB in the cytosol also conferred an additional benefit as no correction factor was required to compensate for differences in compartment volume that affect the fluorescence signal when GSB is diluted into the larger vacuole compartment (e.g. Meyer et al. 2001). Overall, MCB can be considered as a suitable in situ probe for GSH in poplar similar to previous reports for Arabidopsis (Fricker et al. 2000; Gutiérrez-Alcalá et al. 2000; Meyer et al. 2001).

The amount of GSB in a cell should be stoichiometrically equivalent to the amount of GSH once the plateau level of the progress curve for the labelling is reached (Meyer et al. 2001). In poplar leaves, however, a fraction of GSH remained unconjugated even after 2 h incubation with 100 μM MCB. Therefore we propose that an additional GSH pool, inaccessible to labelling with MCB, must be present, with the most likely candidate being the chloroplastic GSH pool. Although the chloroplast envelope of intact plastids was clearly permeable to bimanes only a minor part of the GSH pool in isolated, intact chloroplasts from spinach could be labelled with MCB. The far more reactive MBB analogue labelled the entire chloroplastic GSH pool by non-enzymatic conjugation. This result suggests that plastoplasts lack a GST capable of catalysing the conjugation of low concentrations of MCB to GSH. The size of the putative chloroplastic GSH pool in WT leaves was around 50% of the total extractable pool. Comparable figures have been reported for chloroplastic GSH pool sizes of 50–65% for barley leaves following rapid isolation of intact chloroplasts in non-aqueous media (Smith et al. 1985); however, chloroplastic GSH levels are considerably lower following aqueous extraction methods (Noctor et al. 2002). Although the chloroplast volume has not been estimated for poplar leaves, by analogy with the calculations for wheat leaves presented in Noctor et al. (2002), the chloroplastic GSH concentration would be at least several mM. This contrasts with the direct measurement of cytosolic GSH levels around 0.2–0.3 mM in this study. This implies that there would be at least a 10–20-fold gradient of GSH between the chloroplasts and cytosol in vivo and would also be consistent with the observed active accumulation of GSH by isolated chloroplasts (Noctor et al. 2000, 2002).

Depletion of cytosolic GSH by conjugation to MCB during the assay would accentuate the gradient of GSH between chloroplasts and cytosol during the course of the labelling period; however, a major fraction of plastidic GSH was retained in the chloroplasts under these conditions. It would appear that efflux of GSH from the plastids is relatively slow and cannot cover a sudden increased demand in the cytosol resulting from irreversible conjugation of GSH to MCB (this study) or following herbicide treatment (Gullner et al. 2001). There is, of course, the caveat that most of the in situ assays reported here were conducted in the presence of sodium azide and changes in normal physiology might be anticipated as ATP levels alter. Conversely, the increased levels of cytosolic GSH in the transgenic lines did not result in an increase in the putative chloroplastic pool. Taken together, we infer from these data that chloroplastic GSH levels are relatively independent of cytosolic GSH levels, at least in the short term. Further experiments with independent manipulation of the chloroplastic GSH pool either by treatment with plastid-specific herbicides or by using molecular biology tools for selectively altering the chloroplastic GSH biosynthesis pathway will allow the generation of more direct data on the chloroplastic GSH pool. Similarly, expression of a GST active towards MCB in the chloroplasts in the future might allow direct measurements of this subcellular GSH pool.

The cytosolic GSH concentrations in WT poplar leaves of 0.2–0.3 mM reported here are the first direct measurements of GSH in photosynthetically active tissue. Values are comparable with concentrations estimated by Rennenberg (1982) on theoretical grounds, and Gutiérrez-Alcalá et al. (2000) based on direct in situ measurements in leaf epidermal cells of Arabidopsis. The cytoplasmic concentration of GSH in aerial tissues is consistently lower than concentrations in roots of Arabidopsis or exponentially growing cell cultures, where GSH concentrations of up to 4 mM have been found (Fricker et al. 2000; Meyer et al. 2001). The reason for this difference might be related to specific functions of GSH in maintaining cell divisions in meristematic cells (May et al. 1998, Vernoux et al. 2000).

The uniformity of cytosolic GSH levels between different mature cells both in WT leaves and leaves from poplars over-expressing bacterial γ-ECS in the cytosol is perhaps surprising. Similar analysis of root tissues (Fricker et al.
and different leaf epidermal cell types (Gutiérrez-Alcalá et al. 2000) in Arabidopsis revealed variations in GSH levels between different cell types that, in the latter case, also responded differently to environmental stress. It is even more surprising as GSH levels were similar in both photosynthetic and non-photosynthetic cells, that would be expected to differ both in their ability to synthesize GSH or reduce GSSG, and in the demands that are placed on the GSH pool. Studies of other metabolite levels in individual leaf cells using single cell sampling and analysis (SiCSA) or microdissection have revealed considerable heterogeneity in other metabolites between different cell types (Tomes & Sharrock 2001; Outlaw & Zhang 2001). Separate analysis of bundle-sheath and mesophyll from maize leaves also showed a distinct heterogeneity with assimilatory sulphate reduction being almost exclusively localized in the bundle-sheath cells, whereas formation of GSH takes place predominantly in the mesophyll cells (Burgener et al. 1998).

The even distribution of GSH in poplar leaves may reflect a high degree of symplastic continuity between the different cell types, effectively equilibrating metabolite levels across the tissue, or operation of a common control system that regulates the synthesis pathway in all cells, reducing metabolite levels across the tissue, or operation of a common control system that regulates the synthesis pathway in all cells, and lacks the refinement of comparable techniques to map compartmentalization of GSH metabolism in intact leaves. It will be challenging in the future to combine transgenic approaches with cell-specific expression to understand how fluxes through such metabolic pathways are co-ordinated between cells as well as within cells, and how individual cells marshal their metabolism to respond both to general abiotic stresses and to potentially highly localized biotic stresses.

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